NEUROPROTECTION IN THE HIBERNATING BRAIN:

TISSUE TRAUMA AND GLUTAMATE STUDIED BY MICRODIALYSIS

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A

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ABSTRACT

Hibernation, a natural model of tolerance to "cerebral ischemia", represents a state of pronounced fluctuation in cerebral blood flow where no brain damage occurs. This study systematically investigates the brain tissue response of hibernating and euthermic arctic ground squirrels to CNS trauma, modeled by insertion of microdialysis probes. The effect of glutamate, an excitatory amino acid neurotransmitter, on the cellular response and the origin of the significant amount of glutamate were determined by quantitative microdialysis study.

The present results indicate in euthermic brain tissue a typical inflammatory tissue response evidenced by the presence of activated microglia and astrocytes and the oxidative stress response. However, this response was profoundly suppressed in hibernating animals. Importantly, the progressive increase in $[glu]_{dia}$ is not necessarily associated with the enhanced tissue response observed in euthermic animals and could be avoided by using sterile microdialysis technique, which suggests a microbial origin of glutamate.

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CHAPTER 1

INTRODUCTION

1.1. Stroke and Traumatic Brain Injury

Stroke, the consequence of an interruption of cerebral blood flow, is the third most common cause of death in North America and the leading cause of long-term disability in the United States (National Stroke Association, 1994). Estimates indicate an incidence of approximately 750,000 strokes per year in the United States, with about 4 million survivors who are at high risk of a secondary cardiovascular event. Researchers from the University of Iowa College of Medicine at American Heart Association's 23rd International Joint Conference on Stroke and Cerebral Circulation (Orlando, Fla.) reported that by the year 2050 more than 1 million strokes per year are expected in the United States. Overall, stroke represents a large unmet medical need (Fisher and Bogousslawsky, 1998).

Clinical and experimental studies show that following an acute ischemic stroke, neuronal damage continues to progress for periods of several hours to days (Petito et al., 1987; Heiss et al., 1992; Garcia, 1992). This time period is seen as a window of opportunity to allay neuronal damage. Elevated extracellular concentrations of glutamate following cerebral ischemia are thought to mediate the pathological process that eventually results in cell death (Choi and Rothman, 1990). However, to date, there is no medical treatment approved for stroke beyond the tissue plasminogen activator (t-PA), a

thrombolytic agent restricted to administration within 3 hours after stroke (The NINDS rt-PA Study Group, 1995; Wahlgren, 1997)

Traumatic brain injury (TBI) is the most important cause of death for young adults in the United States (Goldstein, 1990) and the incidence of TBI is increasing. After a severe head injury around 40% of patients will die, and of the remainder, 15% to 70% remain disabled (Bullock and Teasdale, 1990).

The clinical characteristics of TBI include elevated intracranial pressure (ICP) (Lundberg N et al., 1965), reduced cerebral blood flow (particularly focal or regional flow reduced to levels that likely represent ischemia) and decreased cerebral metabolic demands (Bouma et al., 1991; Robertson et al., 1992; Salvant and Muizelaar, 1993). High levels of glutamate have been observed following TBI (Faden et al., 1989). Contemporary treatment strategies for TBI are directed toward treating secondary brain injury which involves multiple biochemical and cellular events, such as elevated excitatory amino acids (eg. glutamate), increases in intracellular calcium, acidosis and free radicals (Bullock and Fujisawa, 1992; Katayama et al., 1990; Kontos, 1989; Hall, 1993; Wolf et al., 1993). However, the mechanisms underlying secondary or delayed cell death, progressing from hours to days after initial insult, still has to be fully elucidated.

1.2. Excitotoxicity

Excitatory amino acids (EAAs) normally function as neurotransmitters but are known to mediate excitotoxicity in high concentrations. Glutamate is such a

neurotransmitter present in excitatory presynaptic terminals throughout the brain. The neurotoxicity of glutamate in the brain was established 30 years ago (Olney and Sharpe, 1969) and supported by a number of studies based on glutamate neurotransmission (Curtis and Johnston, 1974; Choi, 1988).

The human brain depends on its blood supply of oxygen and glucose. Irreversible brain damage occurs in only a few minutes if blood flow is highly reduced or completely interrupted. Such reduction (ischemia) is common in some diseases, such as ischemic stroke, TBI, and a variety of neurological disorders (Choi and Rothman, 1990). The reduction in blood flow and consequent decline in ATP levels lead to membrane depolarization, which in turn results in the opening of voltage-operated calcium channels (VOCCs) and Ca^{2+} influx into the cells (Hass, 1983). This increase in Ca^{2+} presynaptically causes the release of glutamate that acts on its several families of postsynaptic receptors (NMDA, AMPA and kainate), especially NMDA receptors, and results in further influx postsynaptically of Ca^{2+} as well as the mobilization of Ca^{2+} from intracellular stores (Fransden and Schousboe, 1991). It is generally accepted that excitotoxic injury to neurons results from these high levels of intracellular Ca^{2+}, which can overstimulate Ca^{2+}-dependent enzymes, such as phospholipases, proteinase and endonucleases (Sims, 1995). This may lead to immediate or delayed cell death (Lipton and Rosenberg, 1994). Recently it has been revealed that accumulation of intracellular Ca^{2+} plays a pivotal role in both of the two major pathways (apoptosis and necrosis) of ischemic cell death (Zipfel et al., 2000).

The discoveries of increased extracellular levels of glutamate during ischemia (Benveniste et al., 1984) and the beneficial effect of NMDA-receptor antagonists in the model of neurological disorders (Faden et al., 1989; McCulloch et al., 1993) are often used to support the neurotoxicity of glutamate. However, more recently, reports on glutamate neurotoxicity have been subjected to intensive debate (Obrenovitch and Urenjak, 1997). The arguments against the systematic interpretation of glutamate excitotoxicity focus on: 1) increased concentrations of extracellular glutamate should not be taken as a reliable indicator of excitotoxicity; 2) the beneficial effect of glutamate antagonists does not necessarily imply an excitotoxic process (Obrenovitch, 1999).

1.3. Inflammatory Response

The brain has no intrinsic immune system since a very effective blood brain barrier (BBB) system inhibits free access of blood cells to the brain tissue. However, microglia, astrocytes and perivascular cells form a network of potential immune cells throughout the CNS (Berry and Logan, 1999; Gehrmann et al., 1995). CNS damage elicits the activation of both astrocytes and microglia (Streit et al., 1999).

Microglia, the small glial cells of mesodermal origin, derive from monocytes and invade neural tissue just before birth. Microglia are the special name of resting macrophages inside the CNS and they are ubiquitous in every part of the CNS and essential for maintaining normal homeostasis (Berry and Logan, 1999). Although little is known about microglia in the normal CNS, it is obvious that microglia readily become activated in response to CNS injuries or neurodegenerative diseases, including

Alzheimer's disease, Parkinson's disease, multiple sclerosis and AIDs dementia complex. Activation of microglia is indicated by a morphological change from a ramified resting state to an amoeboid activate appearance (Streit et al., 1988; Krentzberg, 1996). They are the primary source of brain macrophages.

The activation of microglia is believed to contribute to neurodegenerative processes through the release of proinflammatory and/or cytotoxic factors, including interleukin-1 (IL-1), turmor necrosis factor-α (TNFα), nitric oxide (NO), reactive oxygen intermediates and quinolinic acid (Chao et al., 1992; Dickson et al., 1993; Lee et al., 1993; Matsumoto et al., 1992; Espey et al., 1997). Furthermore, activation of microglia and subsequent production of proinflammatory and cytotoxic factors have been attributed to increased neurotoxicity (Chao et al., 1992; Dawson et al., 1993).

Microglia activation occurs at a very early stage and often precedes reaction of any other cell type in the event of pathological disturbances (Streit et al., 1988; Gehrmam et al., 1995). Therefore they form the first line of defense and are in control of immune response in the brain. It has been demonstrated that a variety of immunological surface marker molecules are expressed on the surface of activated microglia (Morioka et al., 1992; Kato et al., 1995). With the introduction of lectin histochemistry, the rapid and high specific biotin-avidin marker system made it possible to show both resting and activated microglia by using the lectin RCA-1, a carbohydrate binding protein, as detected with the avidin-biotin peroxidase method (Mannoji et al., 1986).

Astrocytes are ubiquitous in the brain and amount to 20% of the total cell volume of the cerebral cortex. They have multiple functions including the transport of blood

cells and nutrients through the BBB and more importantly, maintain ion homeostasis (Rosenman et al., 1995; Varon and Somjen, 1979). Following CNS injuries, such as trauma, disease or chemical insult, astrocytes become reactive, termed astrogliosis. It is now well established that glial fibrillary acidic protein (GFAP) is the principle intermediate filament in mature astrocytes of CNS and is important in modulating astrocyte motility and shape. Astrogliosis is characterized by rapid synthesis of GFAP and is recognized by immunostaining with GFAP antibody. Therefore, GFAP has been recognized as a standard marker for activated astrocytes (for review see Eng et al., 2000).

1.4. Hibernation and Neuroprotection

Hibernation is a unique physiological condition characterized by a decrease in body temperature, heart and respiration rate, reduction in blood flow and suppression of metabolism. The ability of hibernating mammals to survive without neurological damage during dramatic fluctuations in blood flow represents a natural model of tolerance to cerebral ischemia (Frerichs et al., 1994). Moreover, an *in vitro* study indicated that hibernation is associated with tolerance to deprivation of oxygen and glucose (Frerichs and Hallenbeck, 1998). This increased tolerance may arise from a variety of neuroprotective adaptations. Study of hibernation as a model of tolerance to brain trauma may provide directions for the treatment of neuronal damage in CNS trauma or neurodegenerative diseases.

Hypothermia is the first to be considered as such a neuroprotective aspect. During the period of torpor from several days to weeks, hibernating mammals survive

well at core body temperature around 3 °C, which is harmful to non-hibernating species including humans. Experimental studies provide evidence of an association between hypothermia and reduced neuronal injury after ischemia or TBI (Busto et al., 1987). But data in humans is still limited and controversial (Marion et al., 1997; Clifton et al., 2001).

Leukocytopenia may be another remarkable neuroprotective adaptation. Leukocyte counts in hibernating arctic ground squirrels dramatically decrease to about one tenth euthermic values and rapidly increase to euthermic levels during arousal (Zhou et al., 2001; Toien et al., 2001). Although the mechanism of this dramatic change in leukocytes is not yet fully understood, it appears obvious that leukocyte infiltration at sites of injury promotes cytotoxic reactions (Bowes et al., 1993; Whalen et al., 2000). Thus leukocytopenia may serve a neuroprotective role during hibernation.

Neuronal damage is more than the passive outcome of reducing the oxygen supply for adequate energy production to allow survival. Increasing evidence indicates that much of the damage is mediated by active processes such as production of radicals and other oxidizing chemical species (for review see Love, 1999). Oxidative stress, an imbalance between the production of free radicals and the cellular antioxidant response, also contributes to neurological disorders (Smith et al., 2000). The enhanced antioxidant defense during hibernation may provide another important role in neuroprotection (Drew et al., 1999; Toein et al., 2000). The plasma and CSF concentrations of ascorbate (vitamin C), an antioxidant, increase during the period of hibernation. This may protect brain tissue against oxidative stress associated with the fluctuation of blood flow (Drew et al., 1999). In addition to hypothermia, leukocytopenia and increased antioxidant

defense, some other aspects of hibernation physiology, like the inhibition of protein synthesis (Frerichs et al., 1998), and suppression of metabolism (Storey, 1997), may also contribute to other neuroprotective adaptations. Overall, hibernating species are likely to utilize a combination of physiological adaptations during hibernation to minimize the energy demand and neuronal damage resulting from a variety of pathological disorders. Most single neuroprotective treatment used in clinical trial show no clinical efficacy. This promises new drugs in clinical development which may involve a variety of synergistic adaptations (Walgren et al., 1997).

1.5. Scope and Aims

The broad goal of the work in this thesis is to investigate neuroprotection in hibernating mammals. The main aims in this study are:

1. To test the hypothesis that hibernating brain tissue is tolerant to penetrating brain injury.

2. To test the hypothesis that an increase in extracellular glutamate is necessary for progressive traumatic tissue response observed in euthermic animals in aim 1.

3. To investigate the origin of increased extracellular glutamate associated with traumatic tissue response.

CHAPTER 2

METHODOLOGY

2.1. Animals

Hibernating mammals withstand extreme fluctuation of cerebral blood flow without neurological damage, and are used as a model of natural tolerance to ischemia (Frerichs et al., 1994). Arctic ground squirrels (AGS) are such hibernating species and therefore used in the present studies to investigate neuroprotective functions. AGS were live-trapped in northern Alaska and transported to the Institute of Arctic Biology, University of Alaska Fairbanks. Male and female ground squirrels weighing 650- 840 g were housed individually in a temperature-controlled room (20 - 22°C) with a diet of rodent chow, carrots, apples, sunflower seeds and water ad lid on a 12 h light/dark cycle. All animal experimental protocols were approved by the Institutional Animal Care and Use Committee of the University of Alaska Fairbanks.

2.2. Stereotaxic Surgery

The ground squirrels were fasted for at least 4 h before the surgical insertion of telemetry transmitters (model VM-FH, Minimitter, OR) and guide cannulae (CMA, Acton, MA). Surgery was performed under general anesthesia with halothane (Halocarbon Lab, Riveredge, NJ), induced at 5%, and maintained at 2.5-3% mixed with 100% medical grade O_2 at a flow rate of 1.5 L/ml. Surgery was performed under strictly aseptic conditions (Surgical Packs for Stereotaxic Surgery are listed in appendix I). Body

temperature was kept at 36.5-37.5 °C with a fluid-filled heating pad (Omni medical equipment, Cincinnati, OH) throughout the surgery. Precalibrated, wax-coated telemetry transmitters used to monitor the body temperatures were inserted intraperitoneally. The ground squirrels were placed in a rat stereotaxic frame (Stoelting, Wooddale, IL) and the skull was leveled at the points of ear bar zero (EBZ) + 10.0 mm and EBZ + 30.0 mm. The nose bar was then positioned at – 20.0 mm relative to the value at level head. Guide cannulae, containing the obturators, were stereotaxically implanted with their tips into the right and left striatum (AP = 13.5 mm if Frontal Nasal Suture (FNS) to EBZ < 30 mm, or 14 mm if FNS to EBZ >30 mm, L = ± 3.25mm, D = – 4mm) (all the detail calculation procedures are indicated in the appendix II). Four anchor stainless steel screws (BAS, West Lafaette, IN) were implanted into the skull and secured to the base of the guide cannulae with dental cement (Hygenic repair resin, Hygenic Corp, Akron, Ohio).

To eliminate incidence of infection, in addition to the aseptic surgical technique: 1) animals were treated with antibiotic (enrofloxacin, 5mg/kg, sc) one day before surgery and two days postoperatively; 2) for 10 days post-operation, animals were housed individually in stainless steel cages with nesting cotton but without wood shavings; 3) during this 10 day period, wounds were inspected and cleaned daily with 3% betadine diluted in 0.9% sterile saline.

Following 10 days post-op recovery, the animals, housed individually under a light regime of 12:12 h light: dark at 20-22°C , were transferred to the cold chamber with an ambient temperature of 2-4°C on a 4:20 h light: dark cycle, an environmental condition approximating the winter season. Hibernation was observed using the shavings

added technique. Briefly, the animal was noted to be hibernating if wood shavings placed on their backs were not disturbed for about 24 hours. Body temperature was then monitored by telemetry at least 1 day prior to and throughout the microdialysis experiment to verify hibernating state. Animals that remained active were used as cold-adapted euthermic controls.

2.3. Microdialysis

Microdialysis was first described in the 1970s (Delgado et al., 1972) and has been used extensively in experimental studies in various tissues including brain. It is a well-established technique allowing the measurement of many low-molecular-weight substances in the extracellular space. The method relies on the diffusion of dissolved molecules surrounding a microdialysis probe across a semipermeable dialysis membrane into the probe. Nonetheless, insertion of guide cannulae and microdialysis probes into the brain produce a good model of penetrating brain injury. Therefore, the tissue response around the probe (or cannula) track is comparable to traumatic injury.

Artificial cerebral spinal fluid (aCSF), used for perfusing the microdialysis probes in all the *in vivo* and *in vitro* experiments, contained 124 mM NaCl, 2.7 mM KCl, 1.2 mM $CaCl_2$, 0.85 mM $MgCl_2$, 1.4 mM glucose and 24 mM $NaHCO_3$, adjusted to pH = 7.4, Po_2 = 70 - 80 mm Hg, Pco_2 = 30 - 40 mm Hg by bubbling with 95% N_2 / 5% CO_2. A flow rate of 0.05 or 0.1 μL/min is recommended to investigate the extracellular concentrations of neurotransmitters since such slow flow rate has been determined to yield 100% *in vivo* recovery regardless of body temperature (Osbornet et al., 1999).

2.3.1. I*nVivo* Microdialysis

All *in vivo* microdialysis experiments were performed in the hibernaculum with an ambient temperature of 2-4 °C. Therefore, the perfusion fluid (aCSF) was the same temperature for both euthermic and hibernating groups.

Standard *in vivo* microdialysis technique: In standard microdialysis experiments, microdialysis probes (0.5 mm in diameter with a 4 mm dialyzing membrane; CMA/12, Acton, MA) were soaked in 70% ethanol for 10 min to wash out the glycerol. Using a microperfusion syringe pump, FEP tubings (1.2 µL/100 mm) connected to gas-tight glass syringes (CMA, Acton, MA) were rinsed with 1.0 M HCl at a flow rate of 20 µL/min for 10 min followed by Milli-Q purified H_2O at the same flow rate for another 10 min. The probes were connected with FEP tubing and perfused with freshly prepared aCSF (described above) at a flow rate of 0.6 µL/min for 20 min. The probes were then inserted into the striatum through the guide cannulae while animals were hibernating or euthermic. Euthermic animals were under slight anesthesia with halothane when probes were inserted.

Hourly samples were collected consecutively at a flow rate of 0.6 µL/min for 5 h, then the flow rate was decreased to 0.05 or 0.1 µL/min and an overnight sample was collected for a 15-17 h period. Such slow flow rates have been determined to yield 100% *in vivo* recovery for both hibernating and euthermic body temperatures (Osborne et al., 1999). This perfusion protocol was repeated for 3 days in euthermic and 5 days in

hibernating squirrels. The dialysate samples were frozen and stored at – 80°C until HPLC analyses (see below).

Sterile *in vivo* microdialysis technique: The experimental protocol and the sampling procedure of the sterile microdialysis technique were the same as described in the standard procedure, except that, all components of the microdialysis system were sterilized and the experimental procedure was performed under aseptic conditions. Probes were sterilized with ethylene oxide (Anprolene, Andersen Products, Haw River, NC). Milli-Q purified H_2O, gas-tight glass syringes and FEP tubing as well as other instruments were autoclaved (121°C, 15 psi for 20 min) and aCSF was filter sterilized (0.2 μm, Acrodisc, Pall Corporation, Ann Arbor, MI) using aseptic technique. FEP tubing and probes were rinsed with sterile Milli-Q purified H_2O for 10 min at a flow rate of 20 μL/min followed by aCSF perfusion at a flow rate of 0.6 μL/min for 20 min. Insertion of microdialysis probes and sample collection were performed using sterile technique. Dialysate was collected into autoclaved polypropylene 250 μL centrifuge tubes. Samples were collected for three days in both hibernating and euthermic animals.

Leukocyte counts were performed to assess leukocytopenia during hibernation and to monitor evidence of infection. In operated animals, blood was sampled via toe clip during surgery. Of these animals that remained euthermic, blood was sampled on the first day of the experiment via toe clip and on the last day of experiment via cardiac puncture at the time of euthanasia. In operated animals that entered hibernation blood was sampled via heart puncture at the time of euthanasia. In un-operated control animals

(hibernating and euthermic), blood was sampled via cardiac puncture at the time of euthanasia. Using a microcollection system (unopette), blood was collected into heparinized tubes. The total white blood cells were counted manually (1:100 diluted with 2.86% Glacial Acetic Acid solution) using a hemacytometer or automatically on a Coulter T-890 (Miami, FL) within 24 hours of sampling.

2.3.2. *InVitro* Microdialysis

The *in vitro* sampling procedure was the same as *in vivo* experiments except that using an *in vitro* stand (CMA, Acton, MA), probes were placed in a liquid medium (broth), containing 19 mM glutamine, 34 mM Na_2HPO_4, 33 mM KH_2PO_4, 2.0 mM $MgSO_4$ 50 mg/L ferric ammonium citrate, 45 μM $CaCl_2$, 5.5 mM glucose, and were incubated at 37 °C in a water bath during three days of dialysis.

2.4. HPLC Analyses of Glutamate

Method 1: Dialysates (2 μL) were derivitized with 2 μL of o-phthaldialdehyde reagent (1.0 mL of OPA incomplete, Sigma, mixed with 14 μL mercaptoethanol solution diluted in methanol 1:10) in a 250 μL centrifuge tube. After a 2 minutes reaction time, 3.5 μL of the sample was injected onto a microbore column (Pronexus, Stockholm, Sweden) and separated using a mobile phase of 0.1 M acetate buffer including 10% acetonitrile at pH 6.0. Derivatives of glutamate were detected using a CMA/280 fluorescence detector (CMA, Acton, MA) and recorded on a computer using Chromatography Station for Windows (CSW) software (Pronexus, Stockholm, Sweden).

Method 2: Dialysates (10 μL) were derivitized for 2 min with 10 μL OPA reagent with 1.34 mg o-phthaldialdehyde (EM, Gibbstown, NJ) mixed with 0.75 mL methanol , 0.25 mL 0.1M borate buffer (adjusted pH to 9.5 using 10 M NaOH) and 15 μL mercaptoethanal diluted in methanol 1:10. Then the derivitized dialysates were separated on a 10 cm RP column with guard using a mobile phase of 1.0 M sodium acetate (pH = 6.5, adjusted by acetic acid), 2.5% tetrahydrofuran and 25% methanol, pumped at 0.6 mL/min by a Waters 510 pump. The detector was the same as system 1 and the glutamate peak heights were quantified on a Spectra-Physics 4270 integrator with the retention time (RT) at approximately 3.75 min.

In both HPLC systems, glutamate concentration was quantified by comparing peak height to an external standard. All chemicals of the highest purity available were obtained from Sigma (Louis, MO) unless otherwise noted.

2.5. Histology

After 3 days of microdialysis sampling, the euthermic animals were anesthetized with halothane while hibernators were not initially anesthetized. Rectal temperature was measured using a thermocouple in hibernating or anesthetized euthermic animals and blood was sampled via cardiac puncture. All animals were administered an intracardial injection of ketamine (2 mg/kg) and xylazine (0.6 mg/kg) prior to CSF sampling. Then under deep anesthesia, animals were perfused transcardially with at least 500 ml 0.9% NaCl until clear. Following decapitation, brains were quickly removed and placed overnight in ice-cold methacarn (60% methanol, 30% chloroform, 10% acetic acid) with

gentle shaking, then transferred to 70% isopropyl alcohol and stored at 4°C until paraffin embedding.

Prior to paraffin embedding, the whole brain was trimmed, perpendicular to the probe track, into three parts similar in thickness (Fig.1). Following conventional tissue processing procedure (Citadel 2000 Shandon, Pittsburgh, PA) each part of brain tissue was embedded in paraffin and 6 μm thick, consecutive sections were prepared on a sliding microtome for further analysis. Hematoxylin and eosin (H&E) staining were performed on sections around probes and guide cannulae of hibernating (n = 4 striata) and euthermic (n = 6 striata) tissue to investigate pathological tissue response following traumatic brain injury. Sections were processed with conventional histological techniques to determine the location of the probes. In all cases, probes were found to be located in the striatum.

2.6. Immunocytochemistry (ICC)

The non-aldehyde fixed (methacarn) brain tissue sections of hibernating and euthermic squirrels were hydrated through graded ethanol after deparaffinization with xylene. Endogenous peroxidase activity in the tissue was inhibited by incubation in 3% hydrogen peroxide in methanol for 30 min. Sections were then incubated with 10% normal goat serum (NGS) in Tris-buffered saline (TBS; 50 mM Tris-HCl, 150 mM NaCl, pH=7.6) for 30 min at room temperature to eliminate non-specific binding. All washes and the dilutions of antibody were in 1% NGS in TBS. After washing, the sections were incubated overnight at 4°C with either (i) immunoaffinity purified rabbit polyclonal

antibody against GFAP (1:1000) (glial fibrillary acidic protein, kind gift from Dr. Gambetti), used as an established marker for reactive astrocytes, or (ii) biotinylated RCA-1(1:300) (Vector Laboratories, Inc., Burlingame, CA) that recognizes microglia or (iii) immunoaffinity purified rabbit polyclonal antibody against HO-1 (Stressgen Biotechnologies Corporation, Inc., Victoria, BC, Canada) which is used as an oxidative stress marker. Sections stained by GFAP and HO-1 were then incubated in goat anti-rabbit (ICN, Costa Mesa, CA) antisera for 30 min followed by rabbit-specific peroxidase-antiperoxidase complex for 1 hr (Sternberger Monoclonals Inc. and ICN, Cappel): and the staining was developed using 3.3'-diaminobenzidine (DAB) (DAKO Corp., Carpinteria, CA). Sections treated with RCA-1 were incubated with avidin D peroxidase (Vector Laboratories, Inc., Burlingame, CA) for 1 hr and then developed by DAB. The sections were then dehydrated through ascending ethanol and xylene solutions for mounting.

2.7. Statistics

Dialysate concentrations of glutamate in euthermic and hibernating animals are expressed as means ± SEM. Data collected *in vivo* were analyzed using a 2 x 2, repeated measures ANOVA design followed, when appropriate, by one-way ANOVAs and Tukey post-hoc comparisons. *In vitro* results were analyzed using a one-way repeated measures ANOVA design (SAS for windows, version 8. SAS Institute Inc., Cary, NC). All sample analyses were performed on raw data.

Effects of surgery and hibernation state on white blood cell counts obtained on the last day of dialysis was determined using a 2x2 ANOVA design followed by Tukey post-hoc comparisons. Within animal comparisons across time for white blood cell counts in euthermic animals were made using a repeated measures ANOVA design (SAS for Windows, Version 8. SAS Institute Inc., Cary, NC). The criterion for statistical significance was $p < 0.05$.

CHAPTER 3

RESULTS

3.1. Hibernation, a Model of Neuroprotection

(American Journal of Pathology, 2001 in press)

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Abstract

Hibernation, a natural model of tolerance to "cerebral ischemia", represents a state of pronounced fluctuation in cerebral blood flow where no brain damage occurs. Numerous neuroprotective aspects may contribute in concert to such tolerance. The purpose of this study was to determine if hibernating brain tissue is tolerant to penetrating brain injury modeled by insertion of microdialysis probes. Guide cannulae were surgically implanted in striatum of arctic ground squirrels before any of the animals began to hibernate. Microdialysis probes were then inserted in some animals after they entered hibernation and in others while they remained euthermic. The brain tissue from hibernating and euthermic animals was examined 3 days after implantation of microdialysis probes. Tissue response, indicated by examination of H&E stained tissue sections and immunocytochemical identification of activated microglia, astrocytes and HO-1 immunoreactivity, was dramatically attenuated around probe tracks in hibernating animals compared to euthermic controls. No difference in tissue response around guide cannulae was observed between groups. Further study of the mechanisms underlying neuroprotective aspects of hibernation may lead to novel therapeutic strategies for stroke and traumatic brain injury.

Introduction

Clinical and experimental studies show that following CNS trauma, such as traumatic brain injury (TBI) and acute ischemic stroke, neuronal damage continues to progress after the initial insult (Petito et al., 1987; Heiss et al., 1992; Garcia, 1992; Mclntosh et al., 1998). Ischemia and TBI share several common neurodegenerative mechanisms including excitotoxicity, perturbation of Ca^{2+} homeostasis, inflammation and oxidative stress (Rothman and Olney, 1986; Faden et al., 1989; Vila et al., 2000; Berry and Logan, 1999; Nagayama et al., 2000; Shohami et al., 1997). Furthermore, the pathological events in Alzheimer disease and late onset dementia may be triggered by or, at minimum, exacerbated by, impaired cerebral perfusion originating in the microvasculature and are associated with increased inflammation and oxidative stress (Shi et al., 2000). Despite numerous therapeutic strategies found to reduce ischemia-induced damage in experimental models, only tissue plasminogen activator (TPA) has proven effective in controlled clinical trials (The NINDS rt-PA Study Group, 1995; Wardlaw et al., 1997; Wahlgren, 1997). While TPA has improved prognosis for many stroke patients, progress in the development of effective pharmacotherapies for ischemia, TBI and neurodegenerative disorders has been slow.

Hibernation is a unique physiological condition known best for suppression of metabolism and body temperature which is thought to promote survival during periods of food shortage (Lyman, 1948). Less well recognized are the numerous, potentially neuroprotective aspects of hibernation physiology such as leukocytopenia, immunosuppression, inhibition of protein synthesis, enhanced antioxidant defense and

metabolic suppression (Lyman, 1948; Snapp and Heller, 1981; Sidky rt al., 1969; Mckenna and Musacchia 1968; Frerichs et al., 1994; Frerichs et al., 1998; Gentile et al., 1996; Drew et al., 1999; Toein et al., 2001). Consistent with the hypothesis that hibernation is neuroprotective, Frerichs and Hallenbeck reported increased tolerance to hypoxia/aglycemia in hippocampal slices in *vitro* from hibernating thirteen-lined ground squirrels. Moreover, in previous microdialysis studies in our lab (Osborne et al., 1999), hibernating and euthermic arctic ground squirrels were readily distinguished on the bases of tissue response around the microdialysis probe (Hu et al., 1998). In these previous studies, however, probes were in place longer in hibernating than euthermic animals and sterile microdialysis technique was not followed. The purpose of the present study was therefore to modify these experiments to systematically determine how tolerant hibernating brain tissue is to CNS trauma, *in vivo*, where multiple potentially neuroprotective mechanisms may act in concert.

Microdialysis is a well-established and accepted method for sampling neurotransmitter overflow in awake, freely moving animals. The technique has proven effective in numerous applications where correlations in neurotransmitter release and behavior are verified by pharmacological manipulations (Porkka-Heiskanen et al., 1997; Tanda et al., 1997). Nonetheless, insertion of guide cannulae and microdialysis probes into brain tissue produce stab-like wounds characteristic of traumatic brain injury with associated release of IL-1β (Woodroofe et al., 1991), gliosis (Woodroofe et al., 1991; Benveniste and Diemer, 1987), infiltration of granulocytes (de Lange et al., 1995) and neuronal degeneration (Clapp-Lilly et al., 1999). In the present study we assessed tissue

pathology around microdialysis probes and guide cannulae in euthermic and hibernating arctic ground squirrels to test the hypothesis that hibernation attenuates the post-traumatic tissue response seen with brain injury. Results show marked differences in traumatic tissue response in hibernating versus euthermic animals.

Methods

Surgery

All procedures were approved by the Institutional Animal Care and Use Committee. The animals used in this study were male and female arctic ground squirrels (AGS) weighing 650-840 (g) at the time of surgery. Surgery was performed under general anesthesia with halothane (Halocarbon Lab, Riveredge, NJ), induced at 5%, and maintained at 2.5-3% mixed with 100% medical grade O_2 at a flow rate of 1.5 L/ml. Surgery was performed under strictly aseptic conditions. Telemetry transmitters (model VM-FH, Minimitter, OR) used to monitor core body temperature, were implanted intraperitoneally. Ground squirrels, fasted at least 12 hours prior to the surgery, were secured in a stereotaxic frame. Guide cannulae (CMA, Acton. MA) were stereotaxically positioned above the right and left striatum (AP=13.5 or 14mm, L= ± 3.25mm, D= – 4.0mm) described in detail by Osborne et al. (1999), and were slowly lowered 4mm from the cortical surface. Four anchor stainless steel screws (BAS, West Lafaette, IN) were placed on the skull. Cannulae were secured to the screws with dental cement. Antibiotic

(enrofloxacin 5mg/kg) was given 1 day before surgery and 2 days postoperatively by subcutaneous injection.

Following 10 days post-op recovery, the animals, housed individually under a light regime of 12:12 h light: dark at 20-22°C , were transferred to the cold chamber with an ambient temperature of 2-4°C on a 4:20 h light: dark cycle. Hibernation was observed using the shavings added technique. Briefly, the animal was noted to be hibernating if wood shavings placed on the back remained overnight. Body temperature was then monitored by telemetry at least 1 day prior to and throughout the microdialysis experiment to verify hibernating state.

Microdialysis

Microdialysis procedures and experimental protocol were the same as described in detail by Osborne et al.(1999), except that in the present study, all components of the microdialysis system were sterilized via heat (autoclave), ethylene oxide (Anprolene, Andersen Products, Haw River, NC) or 0.2 μm filtration (Acrodisc, Pall Corporation, Ann Arbor, MI). Briefly, the continuously perfused microdialysis probes (CMA 12/04, O.D. = 0.5mm) were slowly inserted into the right and left striatum through guide cannulae while animals were hibernating or euthermic. Euthermic animals were lightly anesthetized with halothane, induced as described for surgery, maintained at 1% for approximately 5 min. Euthermic animals recovered from anesthesia within about 15 min of inserting probes. Microdialysis probes were perfused by means of a CMA/102 syringe pump with artificial cerebral spinal fluid (aCSF contained 124 mM NaCl, 2.7 mM KCl, 1.2 mM $CaCl_2$, 0.85 mM $MgCl_2$, 1.4 mM glucose, 24 mM $NaHCO_3$, adjusted to pH =

7.4, Po_2 = 70-80 mmHg, Pco_2 = 30-40 mmHg by bubbling with 95% N_2 / 5% CO_2). The perfusion protocol, 0.6 µl/min for 5 h; 0.1 µl/min for overnight, was repeated 3 days in all hibernating and euthermic animals. All microdialysis experiments were performed in the hibernaculum with an ambient temperature of 2-4 °C. Therefore, the perfusion fluid (aCSF) was the same temperature for both euthermic and hibernating groups.

Dialysate collected at 0.6 µl/min during the day or 0.1 µl/min overnight was analyzed for glutamate by High Pressure Liquid Chromatography (HPLC) coupled with fluorescence detection. Dialysates were derivitized with o-phthaldialdehyde reagent [1.0 ml of OPA incomplete (Sigma, St.Louis, MO), mixed with 14ul mercaptoethanol solution diluted in methanol in 1:10] and separated on a microbore column (Pronexus, Stockholm, Sweden) using a mobile phase of 0.1 M acetate buffer including 10% acetonitrile at pH 6.0. Derivatives of glutamate were detected using a CMA/280 fluorescence detector (CMA, Acton, MA) and recorded on the computer via Chromatography Station for Windows (CSW) software (Pronexus, Stockholm, Sweden). Glutamate concentration was quantified by comparing peak height to an external standard.

Total white blood cell counts were performed to assess leukocytopenia during hibernation and to monitor evidence of infection. In operated animals, blood was sampled via toe clip during surgery. Of these animals that remained euthermic, blood was sampled on the first day of the experiment via toe clip and on the last day of experiment via cardiac puncture at the time of euthanasia. In operated animals that entered hibernation blood was sampled via heart puncture at the time of euthanasia. In

un-operated control animals (hibernating and euthermic), blood was sampled via cardiac puncture at the time of euthanasia. Using a microcollection system (unopette), blood was collected into heparinized tubes. The total white blood cells were counted manually (1:100 diluted with 2.86% Glacial Acetic Acid solution) using a hemacytometer or automatically on a Coulter T-890 (Miami, FL) within 24 hours of sampling.

Tissue Preparation

After 3 days of microdialysis sampling, the euthermic animals were anesthetized with halothane while hibernators were not initially anesthetized. Rectal temperature was measured using a thermocouple in hibernating or anesthetized euthermic animals and blood was sampled via cardiac puncture. All animals were administered an intracardial injection of ketamine (2 mg/kg) and xylazine (0.6 mg/kg) prior to CSF sampling as described in Drew et al., (1999). Then under deep anesthesia, animals were perfused transcardially with at least 500 ml 0.9% NaCl until clear. Following decapitation, brains were quickly removed and placed overnight in ice-cold methacarn (60% methanol, 30% chloroform, 10% acetic acid) with gentle shaking, then transferred to 70% isopropyl alcohol and stored at 4°C until paraffin embedding.

Histology

Prior to paraffin embedding, the whole brain was trimmed, perpendicular to the probe track, into three parts similar in thickness (Fig.1). Following conventional tissue processing procedure (Citadel 2000 Shandon, Pittsburgh, PA) each part of brain tissue was embedded in paraffin and 6 μm thick, consecutive sections were prepared on a sliding microtome for further analysis. Hematoxylin and eosin (H&E) staining were

performed on sections around probes and guide cannulae of hibernating (n = 4 striata) and euthermic (n = 6 striata) tissue to investigate pathological tissue response following traumatic brain injury.

Immunocytochemistry (ICC)

The non-aldehyde fixed (methacarn) brain tissue sections of hibernating and euthermic squirrels were hydrated through graded ethanol after deparaffinization with xylene. Endogenous peroxidase activity in the tissue was inhibited by incubation in 3% hydrogen peroxide in methanol for 30 min. Sections were then incubated with 10% normal goat serum (NGS) in Tris-buffered saline (TBS; 50 mM Tris-HCl, 150 mM NaCl, pH=7.6) for 30 min at room temperature to eliminate non-specific binding. All washes and the dilutions of antibody were in 1% NGS in TBS. After washing, the sections were incubated overnight at 4°C with either (i) immunoaffinity purified rabbit polyclonal antibody against GFAP (1:1000) (glial fibrillary acidic protein, kind gift from Dr. Gambetti), used as an established marker for reactive astrocytes, or (ii) biotinylated RCA-1(1:300) (Vector Laboratories, Inc., Burlingame, CA) that recognizes microglia (Mannoji et al., 1986), or (iii) immunoaffinity purified rabbit polyclonal antibody against HO-1 (Stressgen Biotechnologies Corporation, Inc., Victoria, BC, Canada) which is used as an oxidative stress marker. Sections stained by GFAP and HO-1 were then incubated in goat anti-rabbit (ICN, Costa Mesa, CA) antisera for 30 min followed by rabbit-specific peroxidase-antiperoxidase complex for 1 hr (Sternberger Monoclonals Inc. and ICN, Cappel): and the staining was developed using 3.3'-diaminobenzidine (DAB) (DAKO Corp., Carpinteria, CA). Sections treated with RCA-1 were incubated with avidin D

peroxidase (Vector Laboratories, Inc., Burlingame, CA) for 1 hr and then developed by DAB. The sections were then dehydrated through ascending ethanol and xylene solutions for mounting.

Statistics

Effects of surgery and hibernation state on white blood cell counts obtained on the last day of dialysis was determined using a 2x2 ANOVA design followed by Tukey post-hoc comparisons. Within animal comparisons across time for white blood cell counts in euthermic animals and dialysate concentrations of glutamate in euthermic and hibernating animals were made using a repeated measures ANOVA design (SAS for Windows, Version 8. SAS Institute Inc., Cary, NC).

Results

At the time probes were inserted, mean core body temperature (± SD, n=3-4) of euthermic animals was (35.9 ± 1.6 °C) and for hibernating animals was (4.1 ± 0.8 °C). Body temperature of hibernating animals remained low (less than 5 °C) throughout the dialysis sampling period. White blood cell counts decreased from 6357 ± 2739/mm^3 in euthermic animals to 456 ± 98/mm^3 in hibernating animals, (mean ± SD, n=8-10, $p<0.0001$ main effect of state) (Fig. 2). White blood cell counts were similar in the operated groups compared to un-operated control animals ($p=0.31$, main effect of surgery) (Fig. 2). White blood cell counts on the day of surgery and the first and last day of the experiment in euthermic animals also remained stable (mean ± SD; day of surgery,

7583 /mm^3 ± 1443/mm^3; day 1 of dialysis, 8812/mm^3 ± 3071/mm^3; last day of dialysis 7343/mm^3 ± 3360/mm^3). Similar white blood cell counts in operated and un-operated animals and stability of counts during the duration of experimental protocol were consistent with clinical observations that operated animals were free of infection.

Microdialysis probe and guide cannulae tracks were easily distinguished based on position (depth) of placement and a clear difference in track diameter (Fig. 1). Examination of H&E stained tissue sections indicated an obvious response around the guide cannula in all hibernating and euthermic animals with no convincing difference among the cases (not shown). However, importantly, results from the sections with probe tracks showed a clear difference in tissue reaction between hibernating animals and controls (Fig.3). In the hibernating animals there was simply a small space surrounded by slightly pale neural parenchyma. Routine light microscopy showed no other cellular tissue response around the probe track. By contrast, euthermic animals showed a number of histological changes, including reactive astrocytosis, microglial activation, macrophage infiltrate, and axonal swellings.

Activated microglia, identified by using RCA-1 binding and amoeboid shape are clearly seen around the probe tract in euthermic tissue (n = 6 striata, Fig. 4A). In contrast, the probe tract is barely discernable in hibernating tissue (n = 4 striata, Fig. 4B). While resting, ramified microglia are seen throughout the hibernating tissue section, no activated microglia are present around the probe tract. Astrocytosis, also visible in H&E stained sections, was confirmed using antibodies against GFAP. GFAP immunoreactivity was more intense around the probe tracts in euthermic animals

compared to hibernating animals (Fig. 4C,D). Hemeoxygenase-1 (HO-1) immunoreactivity, a marker of oxidative stress response induction, was localized primarily to glial cells and present around the probe tract in euthermic tissue (Fig. 4E). In contrast, HO-1 immunoreactivity was absent in hibernating tissue (Fig. 4F). The concentration of glutamate in the dialysate collected from the striatum at qualitative 0.6 μl/min and 0.1μl/min flow rates, stabilized by day 2 and remained stable over three days of dialysis. No significant difference was observed between euthermic and hibernating animals (data not shown).

Discussion

The present results revealed by histopathological analysis suggest that hibernation is neuroprotective. In euthermic brain tissue the typical inflammatory response was evidenced by i) the presence of activated microglia and astrocytes revealed by RCA-1 binding and GFAP immunoreactivity, and ii) an oxidative stress response identified by an increase in HO-1 immunoreactivity. Both types of responses, however, were profoundly suppressed in hibernating animals.

Traumatic brain injury, induced by insertion of microdialysis probes, induces complex cellular reactions characterized by pathological alternations of neurons and glial cells, and release of inflammatory cytokines (Woodroofe et al., 1991; Benveniste and Diemer, 1987; de Lange et al., 1995; Clapp-Lilly et al., 1999). The glial response is referred to as reactive gliosis. Astrocytes, the major population of glial cells within the CNS, become reactive and increase expression of GFAP within the cytoplasm in

response to brain injury (Mathewson and Berry, 1985; Norenberg, 1994). Microglia, resident macrophages in the normal brain, are ubiquitous and readily transformed from a ramified resting state to an amoeboid activated appearance after trauma (Strite et al., 1988; Kreutzberg, 1996). Release of neurotoxins from reactive glial cells contributes to neurodegeneration and increased neurotoxicity resulting in neuronal cell death (Hayes et al., 1992). The size of the probe tracks in euthermic striata (c.a. 100- 200 μm) were considerably larger than in hibernating striata yet still much smaller than the 500 μm O.D. of the probe. Tissue adjacent to the probe likely dies by necrosis. Subsequent macrophage infiltrate, indicated in H & E staining and activated microglia in euthermic tissue, would then be expected to remove cellular debris, and contribute to the probe track in euthermic tissue (Fig. 3A and Fig. 4A,C). In contrast, less cell death and phagocytosis in hibernating tissue likely prevented the appearance of a well defined probe track (Fig. 3B and Fig. 4B,D). Importantly, a progressive increase in $[glu]_{dia}$ was not associated with or necessary for the enhanced traumatic tissue response observed in the euthermic animals.

The significant degree of protection during hibernation likely results from a combination of factors, considering the number of potentially neuroprotective aspects of hibernation physiology. For example, small decreases in intra-ischemic brain temperature decreases the extent of ischemia-induced neuronal injury (Busto et al., 1987), and moderate hypothermia has been shown to hasten neurological recovery in patients with severe traumatic brain injury (Marion et al., 1997). While hypothermia has been shown to contribute to the neuroprotective state in hibernation (Frerichs and Hallenbeck,

1998), it is unlikely that hypothermia is entirely responsible for the marked difference in brain tissue response.

Profound leukocytopenia, reported here for arctic ground squirrels, is another potentially neuroprotective hallmark of hibernation (Sidky et al., 1969; Frerichs et al., 1994; Toein et al., 2001). Neutrophil adhesion and macrophage infiltration at sites of injury promote cytotoxic reactions (Weiss, 1989; Bowes et al., 1993; Whalen et al., 2000; Buzadzic et al., 1990). Thus leukocytopenia and decreased antibody formation (Mckenna and Musacchia, 1968) may protect hibernating brain tissue during injury. Plasma and cerebral spinal fluid concentrations of ascorbate increase significantly during hibernation (Drew et al., 1999; Toein et al., 2001) as do other antioxidant defense systems (Buzadzic et al., 1990). Increased antioxidant defense systems, therefore, may further protect hibernating brain tissue. The observation that HO-1, an antioxidant protein, is induced in euthermic but not in hibernating brain supports the hypothesis that hibernation minimizes injury-induced oxidative stress.

Inhibition of protein synthesis shown to be neuroprotective in a rodent model of focal ischemia-reperfusion (Du et al., 1996) also occurs during hibernation (Marion et al., 1997; Knight et al., 2000). Thus, a number of potentially neuroprotective adaptations, in addition to profound hypothermia, leukocytopenia and metabolic suppression (Toein et al., 2001), characterize hibernation and likely produce additive or synergistic neuroprotective effects.

The tissue response around the guide cannulae, which were implanted before any of the animals began to hibernate, was not significantly different between animals that

remained euthermic and those that entered hibernation. This strongly suggests that the neuroprotective effect was due to the hibernation state and not to the difference between animals independent of the state of hibernation. While previous work in this lab (Clapp-Lilly et al., 1999) has shown, in rats, that perfusion of microdialysis probes has a tendency to enhance tissue damage, especially within 100 μm from the probe, the difference in tissue damage between dialyzed and non-dialyzed preparations is minor at both the light and ultrastructural level (Clapp-Lilly et al., 1999). This suggests that perfusion of the probe did not contribute appreciably to the degree of tissue response around the probe track. During the 6 to 8 month hibernation season, ground squirrels enter prolonged states of torpor (1-3 weeks) from which they periodically re-warm for brief (24-48 hrs) periods of euthermia. Speculation still exists as to what drives periodic arousal or why it is necessary. However, it is tempting to hypothesize that a combination of adaptations has evolved in parallel with hibernation to protect vulnerable tissues during frequent periodic re-warming. This same combination of adaptations likely accounts for the profound differences in traumatic brain injury reported here. Better understanding of mechanisms and neuroprotective effects of hibernation may ultimately contribute to the development of effective combination therapies for stroke, head trauma and possibly neurodegenerative disease.

In summary, the current study provides evidence that hibernation plays a crucial role in protecting brain tissue against traumatic injury induced by insertion of microdialysis probes. Many neuroprotective aspects of hibernation physiology, such as hypothermia, leukocytopenia, immunosuppression and enhanced antioxidant defense are

associated with tolerance to penetrating brain injury. Further efforts should be directed at identifying the mechanisms that regulate the suppression of cellular functions in hibernation. This could lead to new therapeutic strategies.

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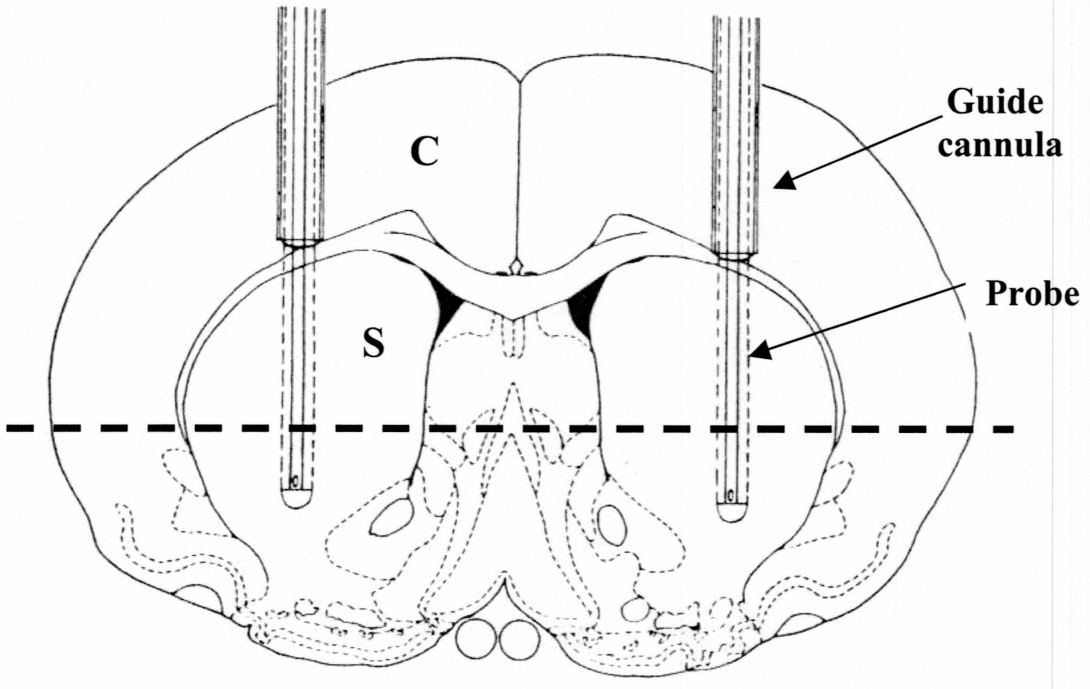

Figure 1: Diagram of coronal view through striatum shows position of guide cannulae (outer dia. = 0.65 mm) and microdialysis probes (outer dia. = 0.5 mm with 4 mm long dialyzing membranes). Following fixation, brains were trimmed perpendicular to the probe tracks as indicated by dashed lines. Sections for histopathological analysis (6μm) were taken through cortex (C) and striatum (S), perpendicular to guide cannulae and microdialysis probe tracks.

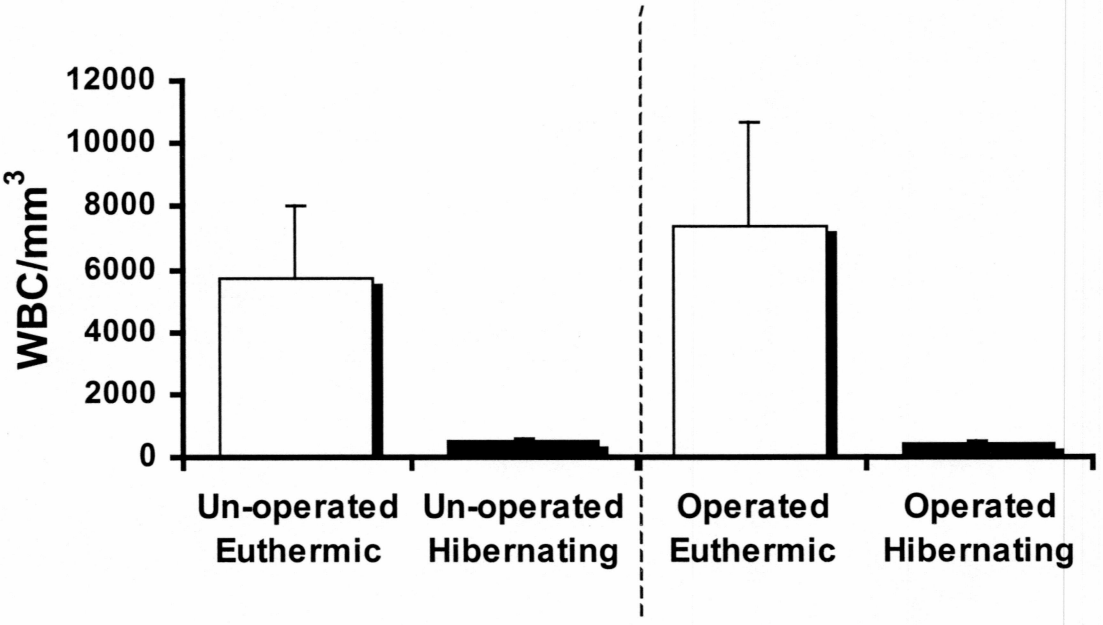

Figure 2: White blood cell (WBC) counts (mean ± SD) for operated and unoperated euthermic and hibernating arctic ground squirrels. Pooled (rectal) body temperature (mean ± SD, n=7-9) for euthermic was (36.46 ± 0.97 °C) and for hibernating was (3.32 ± 1.43 °C).

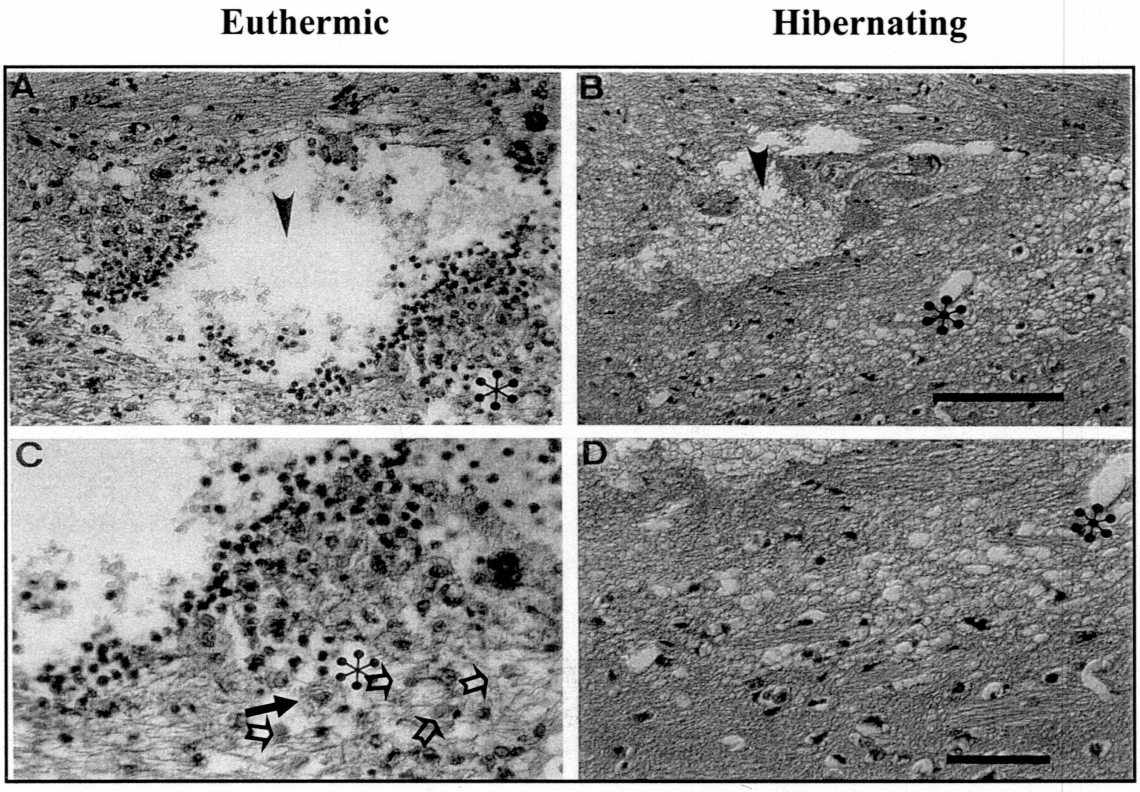
Euthermic
Hibernating
A
B
C
D

Figure 3: H&E stained sections in euthermic animals as compared with hibernating animals. A greater tissue reaction is seen in H&E stained sections in euthermic animals (A, C) as compared with hibernating animals (B, D). C and D are pictures with higher magnification of the area marked with (*) in A and B. Arrowheads point to the probe track. Sections from euthermic animals (A and C) demonstrate the probe cavity, accompanied by mononuclear inflammatory infiltrate, accumulation of lipid-laden macrophages (filled arrow), axonal swellings (open arrows) and fibrillary gliosis. In contrast, brain from hibernating animals (B and D) showed the probe cavity with no discernible inflammatory or reparative reaction. Specifically, no macrophages were seen, and there were no swollen axons or histological evidence of astrocytosis. There was simply a cavity surrounded by slight pallor of the surrounding neural parenchyma. Scale bar: A, B=100 μm, C, D=50 μm.

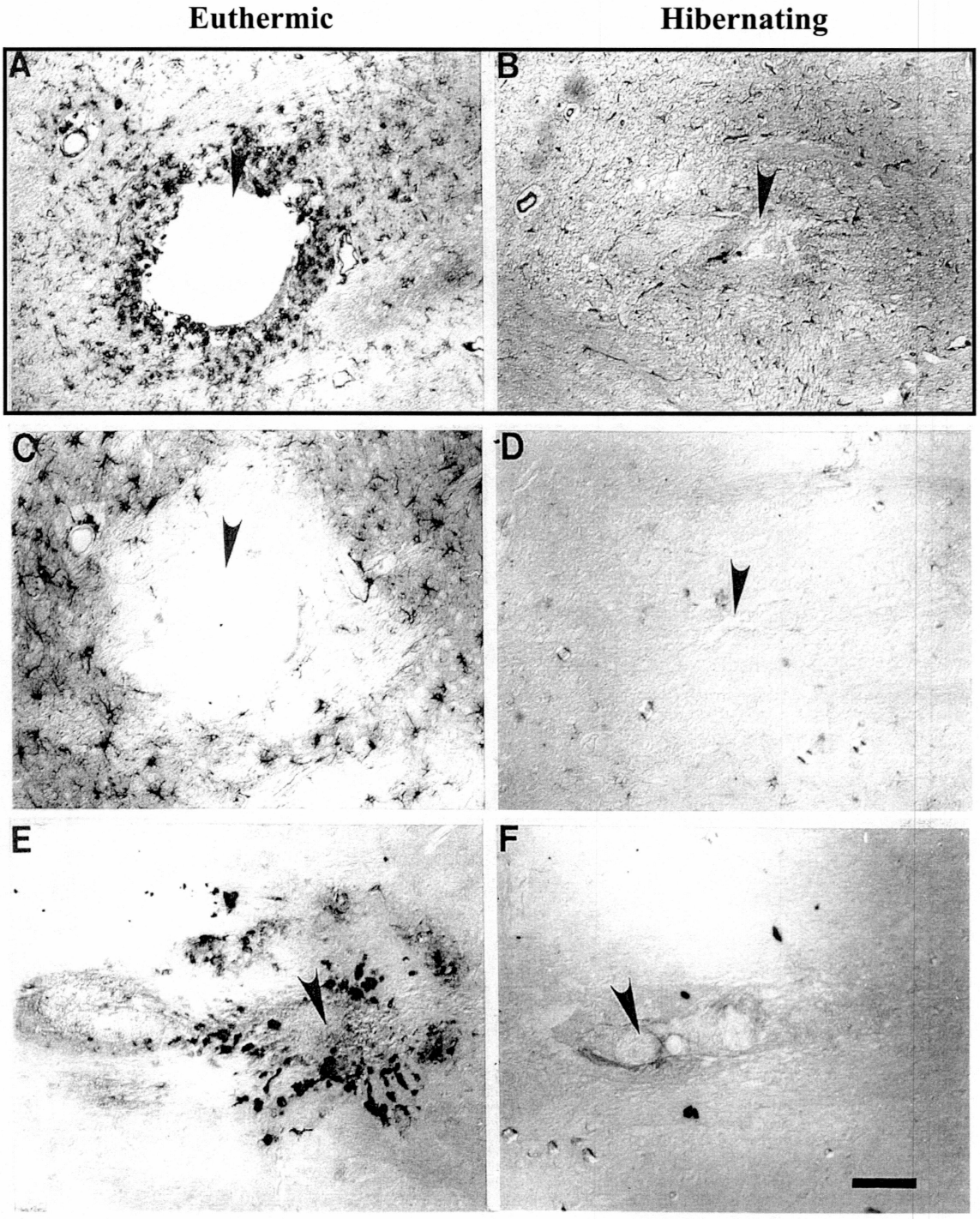
Euthermic
Hibernating
A
B
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Figure. 4: Immunocytochemical identification of activated microglia, astrocytes and HO-1 immunoreactivity. Activated microglia are visible around the probe tract (filled arrow) in euthermic tissue (A). In contrast, the probe tract (filled arrow) is barely discernable in hibernating tissue (B) where only resting (ramified) microglia (arrow) are seen. Scale bar: A,B=100μm. GFAP immunoreactivity around probe tract (filled arrow) is more intense in euthermic tissue (C) than hibernating tissue (D). Scale bar: C,D=100μm. Around probe tract (filled arrows in E and F) HO-1 immunoreactivity is seen in euthermic tissue (E) but is absent in hibernating tissue (F). Scale bar: E,F=100μm.

3.2. Attenuation of Delayed Increase in Glutamate Following Traumatic Brain Injury

(Journal of Neurotrauma, submitted)

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Abstract

Glutamate is an excitotoxin that exacerbates tissue trauma following stroke and traumatic brain injury (TBI). Previous results in our lab showed decreased traumatic tissue response in hibernating compared to euthermic animals. The purpose of the present study was to determine if a progressive increase in glutamate is associated with increased tissue response observed previously in euthermic arctic ground squirrels (AGS). Using quantitative microdialysis in the striatum of AGS, we showed that dialysate glutamate concentrations ($[glu]_{dia}$) progressively increased to around 200 μM after 3 days in euthermic but not hibernating ground squirrels. While greater traumatic tissue response in euthermic animals could account for the increase in $[glu]_{dia}$ we speculated that, given the vastly different body temperatures between the two groups (37°C vs. 4.5°C), glutamate might have originated from microbes, replicating at a faster rate in the warmer animals. To investigate this further, we: i) repeated the *in vivo* experiments with all components of the microdialysis system sterilized using ethylene oxide, heat (autoclave) or 0.2 μm filtration; and, ii) monitored dialysate glutamate concentrations collected *in vitro* from probes immersed in glutamine-rich liquid medium incubated at 37°C. Importantly, using a sterile microdialysis technique, we eliminated the progressive increase in glutamate *in vivo* as well as *in vitro*. These results suggest that microbial contamination can yield significant amounts of glutamate and could play important roles in the pathogenesis of penetrating brain injury and bacterial meningitis.

Introduction

Glutamate, the principal excitatory neurotransmitter, is thought to be a critical factor common to a variety of neurological disorders, including ischemic stroke, traumatic brain injury (TBI) and neurodegenerative disorders (Schwarcz et al., 1981; Benveniste et al., 1984; Choi and Rothman, 1990; Meldrum and Garthwaite, 1990; Zauner and Bullock, 1995). Given the importance of glutamate, measuring the concentration of extracellular glutamate in the animal brain using microdialysis is a major research tool used by many laboratories to monitor extracellular levels of glutamate (Benveniste, 1989). While the technique has proven effective in numerous applications, insertion of microdialysis probes into brain tissue produces stab-like wounds characteristic of traumatic brain injury with associated release of IL-1β (Woodroofe et al., 1991), gliosis (Woodroofe et al., 1991; Benveniste and Diemer, 1987), infiltration of granulocytes (de Lange et al., 1995) and neuronal degeneration (Clapp-Lilly et al., 1999). Previously, we assessed tissue pathology around microdialysis probes in euthermic and hibernating arctic ground squirrels and found that hibernation attenuates the post-traumatic tissue response (Zhou et al., 2001). Given the role of glutamate as an excitotoxin, the purpose of the present study was to determine if an increase in extracellular glutamate is associated with increased tissue response.

Methods

All animal experimental protocols were approved by the Institutional Animal Care and Use Committee of the University of Alaska Fairbanks. Microdialysis was

performed *in vivo* as well as *in vitro* using sterile and standard microdialysis techniques.

Artificial cerebral spinal fluid (aCSF), used for perfusing the microdialysis probes in all the *in vivo* and *in vitro* experiments, contained 124 mM NaCl, 2.7 mM KCl, 1.2 mM $CaCl_2$, 0.85 mM $MgCl_2$, 1.4 mM glucose and 24 mM $NaHCO_3$, adjusted to pH = 7.4, Po_2 = 70 - 80 mm Hg, Pco_2 = 30 - 40 mm Hg by bubbling with 95% N_2 / 5% CO_2.

In vivo microdialysis

Animals and surgery

Arctic ground squirrels were live-trapped in northern Alaska and transported to the Institute of Arctic Biology, University of Alaska Fairbanks. Male and female ground squirrels weighing 650- 840 g were housed individually in a temperature-controlled room (20 - 22°C) with a diet of rodent chow, carrots, apples, sunflower seeds and water ad lid on a 12 h light/dark cycle.

The ground squirrels were fasted for at least 4 h before the surgical insertion of telemetry transmitters (model VM-FH, Minimitter, OR) and guide cannulae (CMA, Acton, MA) as previously described (Osborne et al., 1999; Zhou et al., 2001). General anesthesia induced with halothane (Halocarbon Lab, Riveredge, NJ) at 5% was maintained at 2.5 - 3% mixed with 100% medical grade O_2 at a flow rate of 1.5 L/min. Surgeries were performed under strictly aseptic conditions. Body temperature was kept at 36.5-37.5 °C with a fluid-filled heating pad (Omni medical equipment, Cincinnati, OH) throughout the surgery. Precalibrated, wax-coated telemetry transmitters used to monitor the body temperatures were inserted intraperitoneally. The ground squirrels were placed in a rat stereotaxic frame (Stoelting, Wooddale, IL) and the skull was leveled at the points

of ear bar zero (EBZ) + 10.0 mm and EBZ + 30.0 mm. The nose bar was then positioned at – 20.0 mm relative to the value at level head. Guide cannulae, containing the obturators, were stereotaxically implanted with their tips into the right and left striatum (AP = 13.5 mm if Frontal Nasal Suture (FNS) to EBZ < 30 mm, or 14 mm if FNS to EBZ >30 mm, L = ± 3.25mm, D = – 4mm). Four anchor stainless steel screws (BAS, West Lafaette, IN) were implanted into the skull and secured to the base of the guide cannulae with dental cement (Hygenic repair resin, Hygenic Corp, Akron, OH).

To eliminate incidence of infection, in addition to the aseptic surgical technique: 1) animals were treated with antibiotic (enrofloxacin, 5 mg/kg, sc, Bid) one day before surgery and two days postoperatively; 2) for 10 days post-operation, animals were housed individually in stainless steel cages with nesting cotton but without wood shavings; 3) during this 10 day period, wounds were inspected and cleaned daily with 3% betadine.

After 10 days post-op recovery at 20 - 22°C, animals were transferred to a cold chamber with an ambient temperature of 2 - 4°C on a 4:20 h light/dark cycle, an environmental condition approximating the winter season. Animals were identified to be hibernating by using the previously described shaving added technique (Drew et al., 1999). Body temperature was monitored via telemetry throughout microdialysis experiments. Animals that remained active were used as cold-adapted euthermic controls.

Standard microdialysis technique

In standard microdialysis experiments, microdialysis probes (0.5 mm in diameter with a 4 mm dialyzing membrane; CMA/12, Acton, MA) were soaked in 70% ethanol for 10 min to wash out the glycerol. Using a microperfusion syringe pump, FEP tubing (1.2 μL/100 mm) connected to gas-tight glass syringes (CMA, Acton, MA) were rinsed with 1.0 M HCl at a flow rate of 20 μL/min for 10 min followed by Milli-Q purified H_2O at the same flow rate for another 10 min. The probes were connected with FEP tubing and perfused with freshly prepared aCSF (described above) at a flow rate of 0.6 μL/min for 20 min. The probes were then inserted into the striatum through the guide cannulae while animals were hibernating or euthermic. Euthermic animals were under slight anesthesia with halothane when probes were inserted.

Hourly samples were collected consecutively at a flow rate of 0.6 μL/min for 5 h, then the flow rate was decreased to 0.05 or 0.1 μL/min and an overnight sample was collected for a 15-17 h period. Such slow flow rates have been determined to yield 100% *in vivo* recovery for both hibernating and euthermic body temperatures (Osborne et al., 1999). This perfusion protocol was repeated for 3 days in euthermic and 5 days in hibernating squirrels. The dialysate samples were frozen and stored at $-80^{\circ}C$ until HPLC analyses (see below).

Sterile microdialysis technique

For sterile microdialysis technique, the experimental protocol and the sampling procedure were the same as described in the standard procedure, except that in this study, all components of the microdialysis system were sterilized and the experimental procedure was performed under aseptic conditions. Probes were sterilized with ethylene

oxide (Anprolene, Andersen Products, Haw River, NC). Milli-Q purified H_2O, gas-tight glass syringes and FEP tubing as well as other instruments were autoclaved (121°C, 15 psi for 20 min) and aCSF was filter sterilized (0.2 μm, Acrodisc, Pall Corporation, Ann Arbor, MI) using aseptic technique. FEP tubing and probes were rinsed with sterile Milli-Q purified H_2O for 10 min at a flow rate of 20 μL/min followed by aCSF perfusion at a flow rate of 0.6 μL/min for 20 min. Insertion of microdialysis probes and sample collection were performed using sterile technique. Dialysate samples were collected into autoclaved polypropylene 250 μL centrifuge tubes. Samples were collected for three days in both hibernating and euthermic animals.

Verification of probe placement

At the end of the microdialysis experiments, euthermic animals were anesthetized with halothane while hibernators were not anesthetized. Following decapitation, the brains were removed and fixed in methacarn (60% methanol, 30% chloroform and 10% acetic acid). Sections were processed with conventional histological techniques to determine the location of the probes. In all cases, probes were found to be located in the striatum.

In vitro microdialysis

The *in vitro* sampling procedure was the same as *in vivo* experiments except that using an *in vitro* stand (CMA, Acton, MA), probes were placed in a liquid medium (broth), containing 19 mM glutamine, 34 mM Na_2HPO_4, 33 mM KH_2PO_4, 2.0 mM

$MgSO_4$ 50 mg/L ferric ammonium citrate, 45 μM $CaCl_2$, 5.5 mM glucose, and were incubated at 37 °C in a water bath during three days of dialysis.

Two standard microdialysis techniques were performed: 1) Dialysis system was rinsed with 1.0 M HCl followed by Milli-Q purified H_2O before the aCSF perfusion, exactly as described in *in vivo* standard technique; or, 2) Dialysis system was rinsed with 70% isopropyl alcohol followed by Milli-Q purified H_2O before the aCSF perfusion. The flow rate and the duration for each rinsing period were the same for both standard techniques. The sterile *in vitro* microdialysis procedure was the same as *in vivo* sterile experiments.

HPLC analyses of glutamate

Analyses of $[glu]_{dia}$ was performed by High Pressure Liquid Chromatography (HPLC) using one of the following two systems. System 1: Dialysates (2 μL) were derivitized with 2 μL of o-phthaldialdehyde reagent (1.0 mL of OPA incomplete, Sigma, mixed with 14 μL mercaptoethanol solution diluted in methanol 1:10) in a 250 μL centrifuge tube. After a 2 minutes reaction time, 3.5 μL of the sample was injected onto a microbore column (Pronexus, Stockholm, Sweden) and separated using a mobile phase of 0.1 M acetate buffer including 10% acetonitrile at pH 6.0. Derivatives of glutamate were detected using a CMA/280 fluorescence detector (CMA, Acton, MA) and recorded on a computer using Chromatography Station for Windows (CSW) software (Pronexus, Stockholm, Sweden). System 2: Dialysates (10 μL) were derivitized for 2 min with 10 μL OPA reagent with 1.34 mg o-phthaldialdehyde (EM, Gibbstown, NJ) mixed with 0.75 mL methanol , 0.25 mL 0.1M borate buffer (adjusted pH to 9.5 using 10 M NaOH) and

15 μL mercaptoethanal diluted in methanol 1:10. Then the derivitized dialysates were separated on a 10 cm RP column with guard using a mobile phase of 1.0 M sodium acetate (pH = 6.5, adjusted by acetic acid), 2.5% tetrahydrofuran and 25% methanol, pumped at 0.6 mL/min by a Waters 510 pump. The detector was the same as system 1 and the glutamate peak heights were quantified on a Spectra-Physics 4270 integrator with the retention time (RT) at approximately 3.75 min. In both HPLC systems, glutamate concentration was quantified by comparing peak height to an external standard. All chemicals of the highest purity available were obtained from Sigma (Louis, MO) unless otherwise noted.

Statistics

Data are expressed as means ± SEM. Data collected *in vivo* were analyzed using a 2 x 2, repeated measures ANOVA design followed, when appropriate, by one-way ANOVAs and Tukey post-hoc comparisons. *In vitro* results were analyzed using a one-way repeated measures ANOVA design (SAS for windows, version 8. SAS Institute Inc., Cary, NC). All analyses were performed on raw data. The criterion for statistical significance was $p < 0.05$.

Results

In vivo evaluation

Using the standard microdialysis procedure, $[glu]_{dia}$ collected at the flow rate of 0.1 μL/min increased significantly ($p < 0.05$) in the euthermic arctic ground squirrels

over three days, while $[glu]_{dia}$ remained stable (ranging between 1 to 2 μM for up to five days) in hibernating striatum ($p = 0.1441$; Fig.1 A).

Interestingly, with the sterile microdialysis technique, the increase in $[glu]_{dia}$ (collected at the flow rate of 0.1 μL/min) observed using standard technique in euthermic animals was completely eliminated. There were no significant differences between groups at any time point ($p = 0.6961$). Furthermore, the $[glu]_{dia}$ decreased from day 1 to day 3 ($p < 0.05$) in both hibernating and euthermic animals (Fig.1 B).

With the standard technique, the differences of $[glu]_{dia}$ between groups was also observed in samples collected at 0.6 μL/min with the euthermic $[glu]_{dia}$ higher than the hibernator's (Fig. 2 A). There was no difference between groups or across time in samples collected at 0.6 μL/min with the sterile microdialysis technique (Fig. 2 B).

In vitro evaluation

With HCl flushing of the dialysis system *in vitro*, the $[glu]_{dia}$ increased significantly from day 1 to day 3 ($p = 0.0014$; Fig. 3). Flushing with 70% isopropyl alcohol produces a similar effect ($p = 0.0348$), error bar on day 3 reflects that in 4 out of 7 experiments, $[glu]_{dia}$ decreased from day 2 to day 3 (using HCl 5/5 increased from day 2 to day 3; Fig. 3). The $[glu]_{dia}$ observed in the HCl group and the 70% isopropyl alcohol group corresponded to an increase of 458% and 527% of day 1 $[glu]_{dia}$, respectively. In contrast, the *in vitro* sterile microdialysis technique completely eliminated the increase in glutamate concentration with $[glu]_{dia}$ consistently remaining at the baseline level (Fig. 3). No significant change in $[glu]_{dia}$ was noted within three days of experiments ($p = 0.1940$).

Discussion

In the present study using standard microdialysis technique $[glu]_{dia}$ increased to around 200 μM over three days of dialysis in the striatum of euthermic (core body temperature, mean ± SEM, 36.94 ± 0.53 °C) but not hibernating (3.37 ± 0.03 °C) Arctic ground squirrels. Histological and immunocytochemical analyses showed a greater tissue response around the probe track in euthermic compared to hibernating animals (Zhou et al., 2001), prompting speculation that the increase in $[glu]_{dia}$ was associated with greater tissue response. Given the vastly different body temperatures between the two groups, an alternative explanation was that glutamate originated from microbial contamination introduced by non-sterile microdialysis technique. To address the origin of the delayed increase in $[glu]_{dia}$, the present study measured glutamate overflow in the striatum of hibernating and euthermic ground squirrels as well as *in vitro* using standard and sterile microdialysis techniques. Results demonstrate that the sterile microdialysis technique eliminated a delayed increase in $[glu]_{dia}$ both *in vivo* and *in vitro*. The data indicate that the delayed increase in $[glu]_{dia}$ observed in euthermic animals originated from microbial contamination showing conclusively that microbes can be a significant source of extracellular glutamate.

A number of studies, based on intracerebral microdialysis, have demonstrated that the concentration of glutamate increases immediately after the initial insult (i.e., penetration injury induced by insertion of microdialysis probes) (Nilsson et al., 1994, Obrenovitch and Urenjak, 1997; Faden et al., 1989). This initial increase in $[glu]_{dia}$ was

observed in the present study and is likely due to leakage of cytoplasmic glutamate through damaged plasma membranes.

A delayed increase of extracellular glutamate following traumatic brain injury has also been described. Indeed, glutamate concentrations continue to increase from hours to days after the primary insult (Bullock et al., 1995) and are thought to contribute to progressive tissue damage. Here we show that $[glu]_{dia}$ in striatum of euthermic ground squirrels increased to 194 μM over three days using standard microdialysis technique. However, sterile microdialysis technique abolished this progressive increase in $[glu]_{dia}$. These data thus suggest that glutamate originated from microbes introduced by the non-sterile microdialysis technique. Consistent with *in vivo* results, *in vitro* experiments further confirm the microbial origin of $[glu]_{dia}$. Using non-sterile technique, glutamate concentrations increased *in vitro* in the absence of traditional sources of glutamate including neurons, glia, macrophages or added protein. Furthermore, this increase was abolished using sterile microdialysis technique. This finding raises the possibility that microbial contamination may have confounded earlier microdialysis studies showing progressive increases in $[glu]_{dia}$. Furthermore, these results firmly establish that microbes can serve as a significant source of extracellular glutamate.

In clinical studies, neurological complications develop in up to 30% of patients with bacterial meningitis (Spranger et al., 1996). Furthermore, microbial infections can occur after neurosurgical procedures and have been found to be caused by a variety of organisms (Kaufaman et al., 1990). Microdialysis experiments have shown a close relationship between brain extracellular and cerebrospinal fluid (CSF) concentrations of

excitatory amino acids in animal meningitis (Guerra et al., 1993). Spranger et al. (1996) reported that glutamate concentrations in the CSF of patients with bacterial meningitis were significantly elevated as compared to viral meningitis and non-inflammatory neurological disease, and that a high CSF concentration of glutamate was the best predictor of poor prognosis in long-term clinical outcome. Although monocytes and lymphocytes were found to release substantial amounts of glutamate in these bacterial meningitis studies, the source of the increased concentration of glutamate in the CSF during bacterial meningitis is still not fully understood. The present results suggest that microbes may be a significant source of extracellular glutamate and could contribute to neurodegeneration in bacterial meningitis and penetrating brain injury.

Certain bacterial genera (particularly *Corynebacterium* and *Brevibacterium*) contain species that excrete glutamate under certain growth conditions such as biotin limitation, or following the addition of antibiotics or surfactants. These conditions all lead to disruption of the cell membrane (Atlas, 1995). These organisms have been widely used in the commercial production of glutamate for its use as a flavor enhancer in foods (Glazer and Nikaido, 1995). However, these organisms generally reductively aminate α-ketoglutarate to produce glutamate, a reaction catalyzed by the enzyme glutamate dehydrogenase. This reaction is favored under low oxygen tensions (Glazier and Nikaido, 1995). Glutamate can also be formed via glutamine and α-ketoglutarate in the presence of excess glutamine, a reaction catalyzed by glutamate synthase (White, 1995). Microorganisms, including *Escherichia coli*, may produce high internal concentrations of glutamate as a response to hyperosmotic stress (Neidhardt et al., 1990). None of these

known conditions provides a full explanation for the increased glutamate, especially that seen in the *in vitro* medium under non-sterile conditions in this study, but does provide a basis for microbial production and even significant secretion of glutamate under specific growth conditions.

In conclusion, these data clearly illustrate that the delayed increase in glutamate concentrations observed in striatum of euthermic ground squirrels using standard microdialysis technique is of microbial origin. Microbial glutamate may compromise neuronal health in some neuropathological conditions. Identity of the microbes involved and mechanisms responsible for microbial glutamate release remain areas for further study.

Acknowledgements

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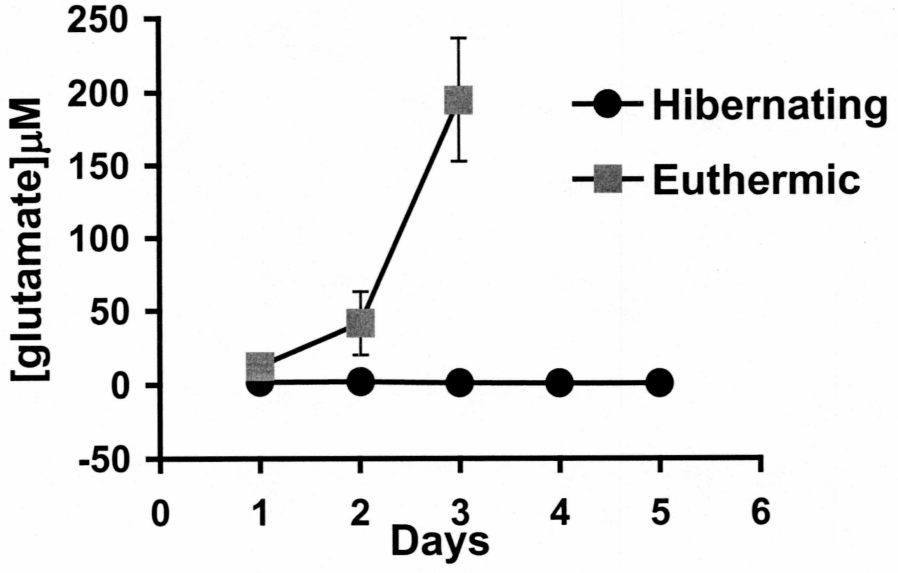
A


250
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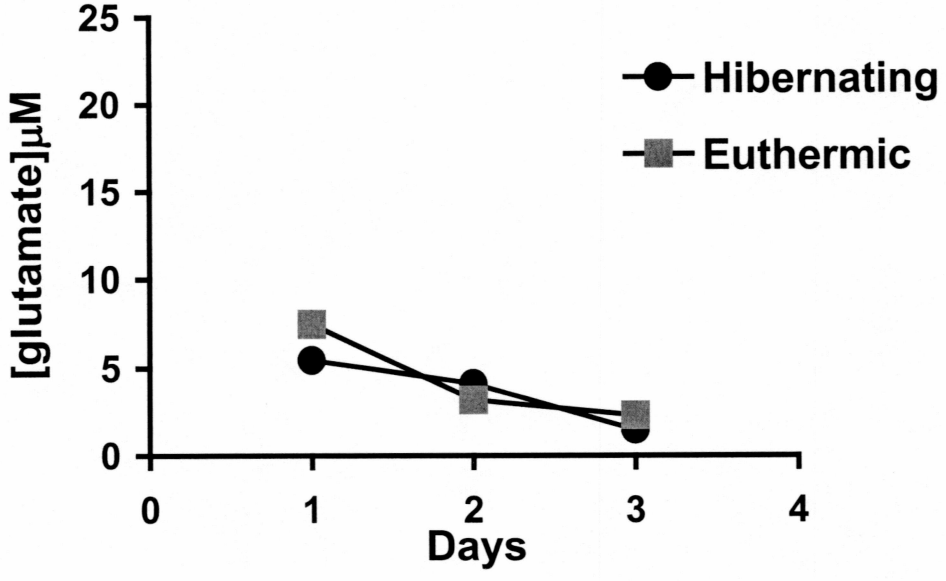
Days
Hibernating
Euthermic

B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4
Days
Hibernating
Euthermic

Figure 5: Quantitative analysis of $[glu]_{dia}$ by using standard and sterile microdialysis technique *in vivo* at the flow rate of 0.10 μL/min. Using standard microdialysis technique and quantitative flow rates (0.05 or 0.10 μL/min) (A), $[glu]_{dia}$ significantly increases over 3 days of dialysis in euthermic striatum ($p < 0.05$, day 3 vs. day 1 and day 2), while $[glu]_{dia}$ remains stable for up to 5 days in hibernating striatum ($p = 0.1441$). The rise in $[glu]_{dia}$ was abolished using sterile microdialysis technique where there were no significant differences between groups (B). Moreover, with the sterile technique, in both groups $[glu]_{dia}$ decreased on day 2 and day 3 compared to day 1 ($p < 0.05$, day 1 vs. day 2 and day 3). Error bars not seen are smaller than the symbol. Core body temperature of hibernating animals remained low (less than 5 °C) throughout the dialysis sampling period and for euthermic animals was (mean ± SEM) 36.63 ± 0.41.

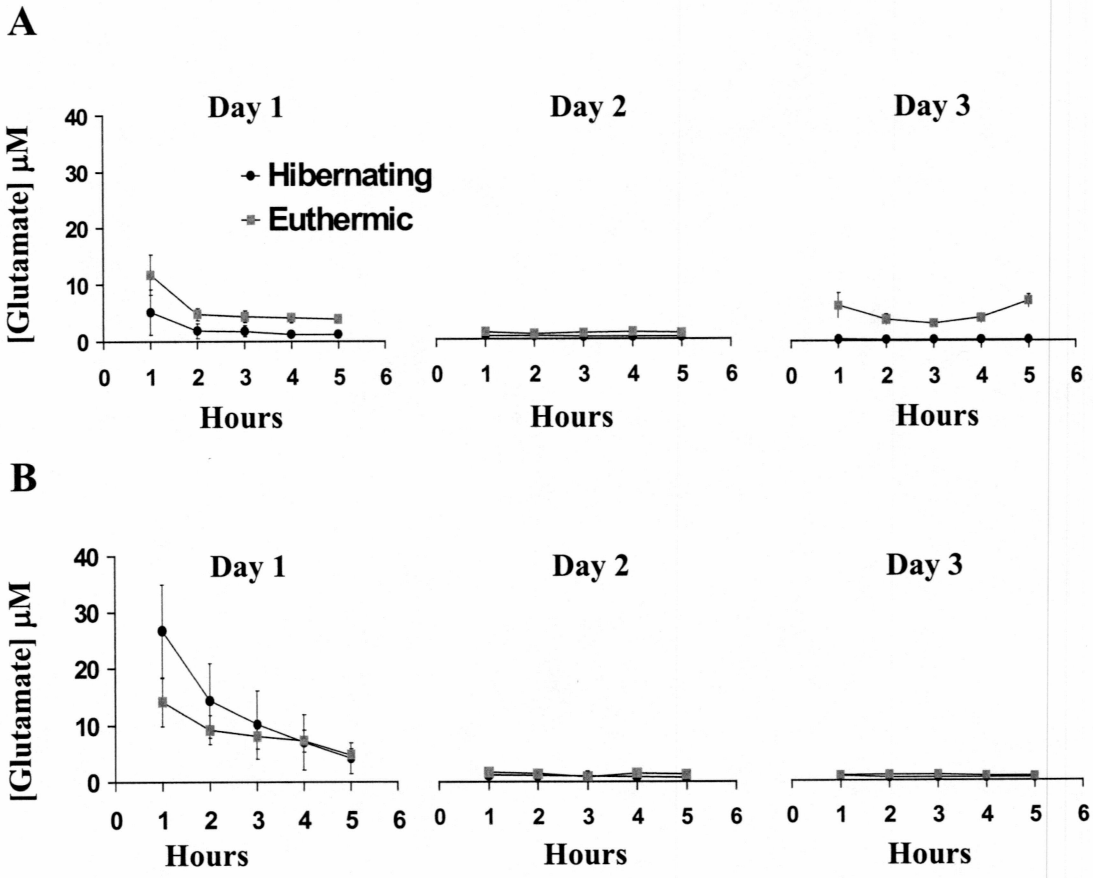
A
Day 1
Day 2
Day 3
Hibernating
Euthermic
[Glutamate] µM
40
30
20
10
0
0 1 2 3 4 5 6
Hours
B
Day 1
Day 2
Day 3
[Glutamate] µM
40
30
20
10
0
0 1 2 3 4 5 6
Hours

Figure 6: Quantitative analysis of $[glu]_{dia}$ by using standard and sterile microdialysis technique *in vivo* at the flow rate of 0.6 μL/min. The increase in $[glu]_{dia}$ was not as pronounced using standard technique (A) but was again attenuated with sterile microdialysis technique (B). Data shown are means ± SEM, n = 4 - 6. Core body temperature of hibernating animals remained low (less than 5 ^{o}C) throughout the dialysis sampling period and for euthermic animals was (mean ± SEM) 36.63 ± 0.41.

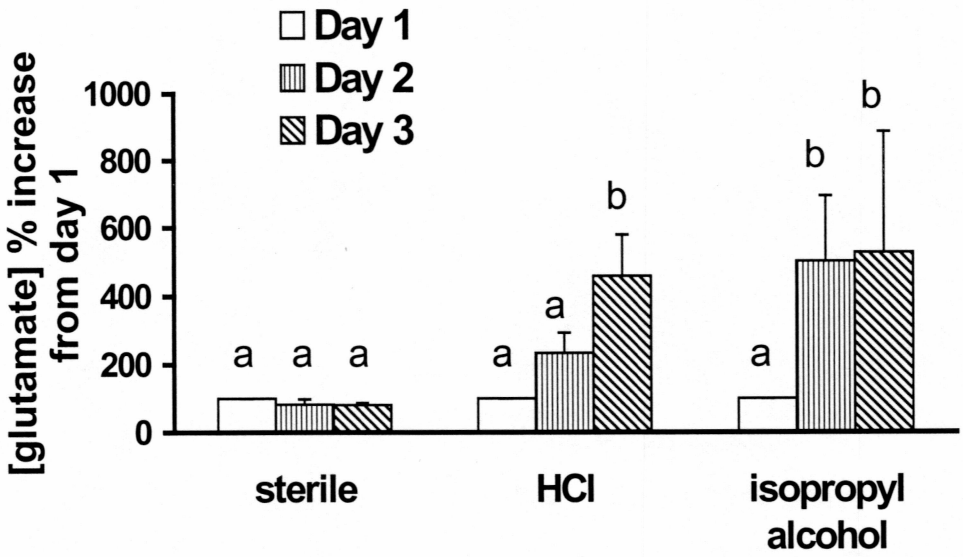

Figure 7. *In vitro* analyses of $[Glu]_{dia}$ by using standard and sterile microdialysis technique. Using standard microdialysis techniques (HCl and 70% isopropyl alcohol) *in vitro*, $[glu]_{dia}$ increased over 3 days when dialyzing tips were immersed in a liquid medium at 36.5-37.5 °C. Letters indicate significant difference ($p < 0.05$). Using sterile technique *in vitro*, no significant differences in $[glu]_{dia}$ were observed within 3 days ($p = 0.1940$). Data shown are means ± SEM, n = 5 - 7 probes. Accumulation of glutamate after day 1 period was defined as 100% (In one set of experiment, the $[glu]_{medium}$ before inoculating bacteria was (mean ± SEM) 24.91 ± 3.88 μM; in another set of experiment, $[glu]_{medium}$ was 1073.30 ± 66.67 μM. Variation of $[glu]_{medium}$ was due to contamination of glutamine with glutamate). Samples were collected at 0.1 μL/min as described for *in vivo* experiments.

CHAPTER 4

CONCLUSIONS

Aim 1 was to test the hypothesis that hibernating tissue is tolerant to penetrating brain injury. By using *in vivo* microdialysis, an excellent model of traumatic brain injury, evidence reported in this study supports the initial hypothesis. Tissue response, indicated by examination of H&E stained tissue sections and immunocytochemical identification of activated microglia, astrocytes and HO-1 immunoreactivity, was dramatically attenuated around probe tracks in hibernating animals compared to euthermic controls.

During hibernation, a number of potentially physiological adaptations, including profound hypothermia, leukocytopenia and increased antioxidant defense, likely produce additive or synergistic neuroprotective effects.

In aim 2, the hypothesis was an increase in $[glu]_{dia}$ is necessary for progressive traumatic tissue response observed in euthermic animals in aim 1. However, the evidence reported in this study suggests that this delayed increase in glutamate is not necessarily associated with enhanced tissue response observed in euthermic tissue. The most interesting finding in addressing aim 3 may be that these significant amounts of glutamate can originate from microbial contamination that should be avoided by using sterile microdialysis technique.

Overall, the studies in this thesis provide evidence that hibernation plays a crucial role in protecting brain tissue against traumatic injury. The delayed increase in $[glu]_{dia}$ characterized as microbial origin is not necessary for the enhanced traumatic tissue

response observed in euthermic compared to hibernating animals. Further efforts should be directed at understanding the mechanisms that regulate the suppression of cellular functions in hibernation as well as identification of the microbes involved and mechanisms responsible for microbial glutamate release.

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Appendix I
Surgical Packs for Stereotaxic Surgery with Transmitter

Guide Cannulae

Gas Sterilize (at least 48 hrs in advance)[1]

- ❑ Guide Cannulae and plug with guide separated from plug (1 or 2/pack)
- ❑ Gel foam cut in ~½ x ½ inch pieces (1/pack)
- ❑ Finger cots (~10/pack)
- ❑ Transmitters (1/pack)

Autoclave in blue drape (Pack #1)

- ❑ 2 guide cannulae holders
- ❑ 2 holders for guide cannulae holders

Abdominal Surgery

Autoclave in blue drape (Pack #2)

- ❑ Scalpel handle (for #15 blades)
- ❑ Retractor (screw type)
- ❑ Blunt scissors or hemostats
- ❑ Drape w/ 2" square tapped off
- ❑ 1" pile of gauze
- ❑ Rat-toothed forceps (med-lg)
- ❑ Needle holder and scissors
- ❑ Fine tipped forceps (straight)
- ❑ Small blunt forceps (straight)
- ❑ Cotton swabs (~4)
- ❑ Prolene 3-0, FS-2 needle (may be re-sterilized)

Stereotaxic Surgery

Autoclave in blue drape (Pack #3)[2]

- ❑ Gauze (1" pile)
- ❑ ~20 cotton swabs
- ❑ Scalpel handle (for #15)
- ❑ Retractor (wire type)
- ❑ Curved forceps (4")
- ❑ Fine tipped forceps (5 INOX, covered w/ pipet tip, 4")
- ❑ Small drill bit to fit screws (bore type)
- ❑ Trephine drill bit (1" dia)
- ❑ Screws (CMA or BAS)
- ❑ 26 ga needle (bent)
- ❑ Twisted tips of Kim wipes
- ❑ Paper clip (formed) for back of head stage
- ❑ Pencils
- ❑ Screw driver that will fit screws
- ❑ Ceramic bowl
- ❑ Spatula
- ❑ Eye dropper
- ❑ (2) Curved hemostats

Closing

Autoclave in blue drape (Pack #4)

- ❑ 3-0 Prolene, FS-2 cutting needle (if left over).
- ❑ Hemostats or needle holder
- ❑ Blunt (straight or curved) forceps, 4")
- ❑ Teflon tape
- ❑ Scissors
- ❑ Cotton swabs
- ❑ Gauze

Extra Stuff

- ❑ Surgery log sheet
- ❑ Anesthesia sheet
- ❑ (2) #15 scalpel blades
- ❑ Full halothane vaporizor
- ❑ O_2 tank
- ❑ Halothane jar with lid fit with gas adapter
- ❑ Nose cone for gas
- ❑ H_2O heating blanket
- ❑ 2 towels
- ❑ Clippers, brush and spray
- ❑ Gas sterilized transmitter
- ❑ Betadine (full strength)
- ❑ 70-75% isopropyl alcohol
- ❑ Cotton gauze
- ❑ (4 packs) Sterile gloves
- ❑ (2 packs) Prolene (3-0, FS-2 cutting needle) for skin
- ❑ Chromic gut (T-5, 1 mm needle) for muscle
- ❑ Dexon (3-0, CE-4, 19 mm needle)
- ❑ 2% betadine
- ❑ nitrofurazone
- ❑ Tape
- ❑ Digital thermometer
- ❑ Eye lubricant (Lube-ide)
- ❑ Clear drape (Baxter)
- ❑ Nose cone for stereotax
- ❑ Stereotax w/ 2 arms
- ❑ Ear bars for stereotax
- ❑ Dental cement (Stoelting)

Chloramphenicol

- ❑ Sterile saline
- ❑ 1 cc syringes w/ needles (26-25 ga)
- ❑ Clear cellophane tape
- ❑ Surgery sheet and pencil
- ❑ Calculator
- ❑ Dremel tool and variac (Be sure drill bits fit in dremel tool)

[1] Gas sterilize using ethylene oxide at least 48 hrs in advance, but no more than 30 days in advance.

[2] Mark a square (~5 x5 inch) in center of drape with autoclave tape

Appendix II

Fast overnight or at least 4 hrs prior to surgery. Baytril, BID beginning 24 hr prior to surgery for a total of 3 days (6 injections)		
Animal ID:	**Weight:**	**Transmitter #:**
Date:	**Sex:**	**Head length (EBZ-FNS):**

Circle side of stereotax used for coordinates: *Left* or *Right*

Record coordinates for Ear Bar Zero (EBZ) before animal is in stereotax.

Record coordinates for Frontal Nasal Suture (FNS) after FNS is exposed

	Anterior Posterior (AP)	**Lateral (L)**
FNZ		
EBZ		
$AP_{FNS-EBZ}$		

Be sure probe holders don't bump.

If FNS-EBZ > 30 mm use +14.0 for AP coordinate; If FNS-EBZ < 30 mm use +13.5 for AP coordinate

Level Head at AP_{EBZ} +10 and +30 to within *0.1 mm*

	AP_{EBZ}+10	AP_{EBZ}+30		AP_{EBZ}+10	AP_{EBZ}+30
1. Dorsal coordinate			5. Dorsal coordinate		
2. Dorsal coordinate			6. Dorsal coordinate		
3. Dorsal coordinate			7. Dorsal coordinate		
4. Dorsal coordinate			8. Dorsal coordinate		

Record nose bar at level skull: _____

Subtract 20 mm -20 mm

Drop nose bar to level skull – 20mm

Nose bar dropped to: _____

Calculate coordinates for right and left guide cannulae: Mark sites with pencile

Coordinates of Pencil marks	**Left**	**Right**	**Coordinates on opposite side at mark**
AP_{EBZ} +13.5 or 14mm	+_____	**AP_{EBZ}** +13.5 or 14mm +_____	
L_{EBZ} ± 3.25 mm	_____	**L_{EBZ}** ± 3.25 mm _____	
$D_{brain\ surface}$ -4.00mm	-_____	**$D_{brain\ surface}$** -4.00mm -_____	

Bring head to level position, Drill holes and insert screws, Drill holes for guide cannulae, Drop nose – 20 mm, insert and cement guide cannulae in place. Administer Enrofloxacin (Baytril), 5mg/kg, sc back of neck, two times a day for three days beginning 24 hrs before surgery. Clip toenails and apply nitrofurazone. Clean surgical site(s) with 3% betadine once/day for 10 days.

Note: if lateral coordinate at EBZ is not on the midline, lateral position of guide cannulae will be relative to midline suture visible on the skull surface at the AP extent of EBZ.